STUDIES ON A CORTICAL LAYER RESPONSE TO STIMULATING AGENTS IN THE ARBACIA EGG

IV. Response to Chemical and Physical Agents in the Absence of Oxygen, and Observations of the Effects of Low Oxygen Tensions and High Hydrostatic Pressures upon Amoeboid Eggs

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Introduction

In response to insemination, or to treatment with certain chemical and physical agents, the cortical layer of the *Arbacia* egg undergoes characteristic irreversible changes (Moser, 1939*a*, 1939*b*). A thin layer of cortical granules breaks down and elevation of the fertilization membrane follows immediately. An interpretation of this and of other related phenomena has been suggested (Moser, 1939*a*, 1939*b*) in terms of the more general theory of stimulation which Heilbrunn and his students have developed (Heilbrunn and Daugherty, 1933; Heilbrunn, 1937).

The experiments described in this paper have been in part devoted to an investigation of the cortical response in the absence of oxygen (see also Moser and Kitching, 1939). *Arbacia* sperm is immobilized in the absence of oxygen, so that fertilization and the resulting membrane elevation do not occur (E. B. Harvey, 1930; Barron, 1932). However, it seemed possible that the cortical response might take place without oxygen if the eggs were subjected to an adequate stimulus. We have therefore investigated, in the absence of oxygen, the effects of certain chemical and physical agents which in air are known (Moser, 1937, 1939*b*) to produce the cortical response.

It is already well established (Loeb, 1895; E. B. Harvey, 1927; Amberson, 1928) that cleavage cannot take place without oxygen or at very low oxygen tensions. However, in view of the definite relationship which seems to exist between the initial cortical response and the subsequent cleavage of the egg (see, for example, Loeb, 1915*a*; R. S. Lillie,

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1915; F. R. Lillie, 1919; Chambers, 1921*a*, 1921*b*; Just, 1919, 1923; and Moser, 1939*b*), we have tried to determine whether the cortical response initiated in the complete absence of oxygen could lead to cleavage when the eggs have been returned to air.

Unfertilized *Arbacia* eggs placed in isotonic urea solution become aspherical and angular in outline within a few minutes and later undergo a peculiar form of amoeboid movement (Moser, 1940; see also Churney, 1940 for amoeboid movements of fertilized *Arbacia* eggs). These movements are mostly rotatory, although sometimes short distances are traversed. *Amoeba proteus* (Hulpieu, 1930) and marine limacine amoebae (Pantin, 1930) slow down and finally stop in the absence of oxygen; and there is recovery in air. The stoppage of movement is almost immediate in the marine amoeba *Flabellula mira* (Kitching, 1939*b*). The amoeboid movement of *Arbacia* eggs is an abnormal activity of a cell whose normal function is cleavage. It seemed, therefore, to be of interest to compare the effects of low oxygen tensions on the normal cleavage and on the amoeboid movement of *Arbacia* eggs.

In addition high hydrostatic pressure inhibits furrow formation of *Arbacia* eggs (Marsland, 1938, 1939) and amoeboid movement of amoebae (Marsland and Brown, 1936). The action of high hydrostatic pressure is attributed to a liquefaction of gelated cortical protoplasm. We have investigated the effect of this agent on the amoeboid movement of unfertilized *Arbacia* eggs treated with urea. We are grateful to Mr. Daniel C. Pease, of Princeton University, for placing his pressure apparatus at our disposal.

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Methods

The general procedure was to subject *Arbacia* eggs under anoxic conditions to treatment which in air is known to elevate fertilization membranes. In addition some observations at various known oxygen tensions were made on the amoeboid movements of eggs in urea, and on cleavage. Eggs therefore had to be kept under microscopical observation while exposed to suitable gas mixtures; and transfer from one solution to another had to be accomplished without leakage of gas to or from the exterior.

Arbacia eggs were obtained in the usual manner. The percentage of membrane elevation after normal insemination was determined for each batch of eggs. Only those batches which yielded 97 per cent to 100 per cent membranes were used in these experiments.

A special glass observation chamber was used (Fig. 1). Besides the usual inlet and outlet for gas, there was a wide bent side-arm which allowed free movement of an iron wire actuated by a Chambers' micromanipulator, but which when filled with mercury did not allow any interchange of gas between the chamber and the outside. To the end of the wire which projected into the chamber was attached with de Khotinsky cement either a micro-needle or a fine glass loop. A cover slip was

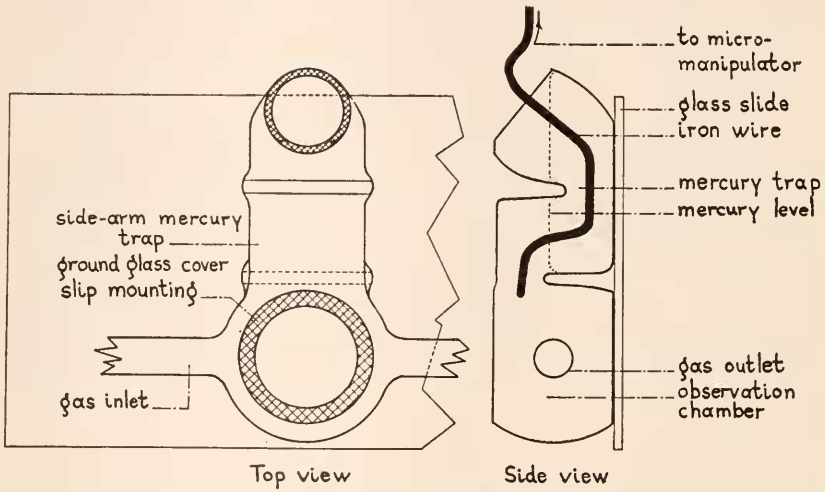

FIG. 1. Drawing of special glass observation chamber (ca. × 1).

sealed with vaseline over the chamber, and eggs could be transferred by means of the loop from one hanging drop to another. In this way eggs could be subjected to a fairly complicated treatment. When several hanging drops were required the cover slip was usually coated with a monolayer of ferric stearate to prevent coalescence, as suggested to us by Mr. R. Ballentine. For this purpose one drop of ferric stearate in benzene was evaporated on the cover slip; and the cover slip was then heated sufficiently to melt the ferric stearate, and finally wiped as clean as possible. The gas leads to and from the chamber were of reasonably flexible lead tubing, so that the chamber could be actuated by the mechanical stage of the microscope, and could also be shaken sufficiently vigorously to make the hanging drops flow together.

The chamber was flushed either with hydrogen purified over platinized asbestos, or with known mixtures of purified hydrogen and oxygen. The apparatus for this purpose is described elsewhere (Kitching, 1939*a*). The gas flowing out from the observation chamber was carried by lead tubing to a water trap.

Another chamber for control experiments was flushed with carbon dioxide-free air. In this way the possible influence of alkalinity of the sea water, due to loss of carbon dioxide, was taken into account.

A hydrostatic pressure apparatus with modifications introduced by Pease and Kitching (1939) was used in the experiments with amoeboid *Arbacia* eggs. Concurrent observations were made on eggs at atmospheric pressure and on eggs in the pressure chamber at high pressure.

Results

Membrane Elevation in the Absence of Oxygen

Saponin.—In each of four experiments a thin hanging drop of sea water containing unfertilized *Arbacia* eggs was placed close to a drop of 0.25 per cent saponin in sea water. The cover slip was mounted over the observation chamber, and pure hydrogen was flushed through continuously at 50–75 cc. per minute. After an interval ranging from 25 to 100 minutes the chamber was shaken (with the hydrogen still running) until the drops flowed together. Membrane elevation occurred within two or three minutes, just as in control eggs similarly treated in carbon dioxide-free air. The times taken for the surface reaction to occur, as shown by a roughening of the surface (see Moser, 1939*b*), for the beginning of elevation, and for the satisfactory elevation of a good membrane, were alike in experimental and control eggs. In both cases cytolysis occurred after some minutes.

Urea.—The effects of isotonic urea upon unfertilized *Arbacia* eggs in air have been described by Moser (1937, 1940). In two experiments a hanging drop of unfertilized eggs in sea water was surrounded by several drops or by a ring of 1.0 molar urea. In this way the ratio of urea to sea water was made large without the disadvantages of a thick drop. After equilibration with pure hydrogen (for seventy minutes in one experiment and eighty in the other) the drops were mixed. Membrane elevation started within fifteen seconds, and within two or three minutes the membranes shrank and disappeared. The eggs were, however, distinguishable by their minutely rough surface from normal unfertilized eggs. Similar results were obtained with eggs treated in air and carbon dioxide-free air. These results are confirmed by later experiments described below.

Sucrose.—In one experiment with sucrose, set up and carried out just as in the case of the experiments with urea, the surface reaction took place without oxygen within twenty seconds, but no membranes were visible. Some of the eggs showed characteristic amoeboid movement (see below) when transferred to carbon dioxide-free air.

Hypertonic Sea Water.—In air, treatment with hypertonic sea water for suitable times, followed by return to ordinary sea water, leads to the appearance of a fertilization membrane (Loeb, 1913). E. B. Harvey (1936) has used 30 grams of sodium chloride in one liter of sea water, and we have followed her formula. In two experiments, after suitable equilibration with hydrogen, unfertilized eggs were transferred with the loop first to hypertonic sea water for about twenty minutes and then to ordinary sea water. A peculiar alveolar structure developed in the cortex. This phenomenon has already been described by Hunter (1936). Similar treatment in carbon dioxide-free air led to membrane elevation in some eggs and to cytolysis in others. Eggs treated in the absence of oxygen first with urea, then with ordinary sea water and finally with hypertonic sea water, did not develop the alveolar cortex, although a number cytolyzed.

Puncture.—When a centrifuged unfertilized *Arbacia* egg is pricked with a micro-needle at any point over the centripetal pole, a characteristic wave of cortical granule breakdown, accompanied by membrane elevation, spreads over the surface (Moser, 1939*b*). Cytolysis rapidly ensues. A number of eggs punctured in hydrogen, and others in carbon dioxide-free air, gave the typical response.

Cleavage and Amoeboid Eggs

Cleavage in Air after Anoxic Chemical Treatment.—If unfertilized *Arbacia* eggs are transferred first to urea for a few minutes, and then back to sea water, the cortical reaction is followed in three or four hours by an irregular cleavage (Moser, 1937, 1940). In these experiments unfertilized eggs after equilibration for an hour in pure hydrogen were transferred to urea for three minutes, and then to three successive washes of sea water; each of the washings lasted for ten minutes. After the eggs had been in the third drop of sea water for ten minutes, the cover slip was placed over an observation chamber in air. Cleavage then occurred in from two to five hours. Although cleavage was irregular, there was no doubt as to its genuine nature, for the nuclei of the daughter cells were clearly visible. The eggs which had remained in the drop of urea solution (having fallen out of the loop) became irregular and finally underwent characteristic amoeboid movement (see

below). In another experiment the eggs were treated in the same manner as those described above, but were not transferred to air; no cleavage or amoeboid movement occurred in these eggs.

The Effect of Low Oxygen Tensions on Cleavage and Amoeboid Movement.—A series of experiments were carried out in which unfertilized eggs in urea and fertilized eggs in sea water were set side by side in two hanging drops on the same cover slip. The drops were set up as soon as possible after treatment with urea or insemination, and exposed over the observation chamber to known mixtures of hydrogen and

TABLE I

The effects of various oxygen tensions upon cleavage in sea water and upon amoeboid *Arbacia* eggs in urea solution. (Room temperature 21.9–24.4° C.)

Experimental Eggs			Control Eggs (in air)	
Oxygen tension in mm. Hg	Cleavage in sea water	Amoeboid movement in urea solution	Cleavage in sea water	Amoeboid movement in urea solution
12.6	Normal	†Active	Normal	Active
6.4	Normal	Active	Normal	Active
4.4	Delayed	Active	Normal	Active
0.95	Eggs wrinkled; no cleavage; finally cytolysis.	Active	Normal	Active
0.42	Eggs wrinkled; no cleavage; finally cytolysis.	A few eggs exhibit very slow amoeboid movement.	Normal	Active
*0.00	Eggs wrinkled; no cleavage.	—	Normal	—

* Pure nitrogen and not hydrogen was used in this experiment.

† The term "active" indicates that all or nearly all of the eggs exhibit the rather sudden changes in shape which have been characterized by Moser (1940, pp. 75–77).

oxygen. Control experiments were carried simultaneously in carbon dioxide-free air and in ordinary air. The results are shown in Table I. At 4.4 mm. of oxygen cleavage was delayed as compared with control eggs, while at 0.95 mm. the eggs entirely failed to cleave. We have not attempted to determine accurately the minimal tension at which cleavage can occur, but this has already been given by Amberson (1928) as 4 mm. Active amoeboid movement took place at oxygen tensions too low for cleavage, and some slight movement was even seen at 0.42 mm. oxygen, while at 0.95 mm. amoeboid movement was active. Fertilized eggs became somewhat wrinkled and shrunken in appearance (as

described by E. B. Harvey, 1927) at oxygen tensions too low for cleavage, or in pure hydrogen. In one experiment a similar result was obtained in pure nitrogen.

Amoeboid eggs in urea solution, as already shown by Moser (1940), finally cease movement; and clear vesicles or "blebs" of various sizes develop and protrude from the surface. This happened in our experiments also, both in air and in mixtures of oxygen and hydrogen. However, in pure hydrogen (in which the eggs remained spherical) the blebs did not develop. Instead a clear layer of uniform thickness formed between the surface membrane and the granular cytoplasm. This layer presumably corresponds to the clear vesicles which develop on amoeboid eggs, and in fact somewhat intermediate clear areas formed in eggs in which movement, as judged by departure from the spherical form, had been only very slight.

TABLE II

The effect of various high hydrostatic pressures upon the amoeboid movement of *Arbacia* eggs. (Room temperature 24.3–26.5° C.)

Pressure		Movements	
lbs./in.²	Atmospheres	Experimental eggs	Control eggs
5,000	340	None	Active movement
3,500*	238	None	Active movement
2,000	136	Some eggs slightly aspherical, but no visible movement.	Active movement
1,000	68	Some active movement.	Active movement

* These eggs were finally returned to atmospheric pressure, after which they became angular within six minutes, and amoeboid within fourteen minutes.

In another experiment unfertilized eggs were treated with urea, by mixing of drops as already described (page 83). Eggs from the same female were treated simultaneously in hydrogen and in carbon dioxide-free air. No movement took place in hydrogen, while active movement occurred in carbon dioxide-free air. The cover slips were then interchanged; and the eggs originally in carbon dioxide-free air stopped movement in pure hydrogen, while those originally in hydrogen began to move quite well in carbon dioxide-free air.

The Effect of High Hydrostatic Pressure on the Amoeboid Movement of Arbacia *Eggs*

In one experiment unfertilized urea-treated eggs were mounted in the pressure chamber, and left at atmospheric pressure until active move-

ment was in progress. The pressure was then raised to 5,000 lbs/in.^{2} (340 atmospheres). Movement ceased in most of the eggs and was only slight in the remainder. Moreover, the outline of all of the eggs became less angular. Accordingly a series of experiments was undertaken in which unfertilized eggs were mounted in the pressure chamber as quickly as possible after treatment with urea, and raised immediately to the desired pressure. The results are shown in Table II. In control experiments urea-treated eggs were observed concurrently at atmospheric pressure.

The effects of high hydrostatic pressure on unfertilized eggs in sea water was investigated in one experiment. Eggs were compressed to 7,000 lbs/in.^{2} (476 atmospheres) for five minutes and then raised to 10,000 lbs/in.^{2} (680 atmospheres) for an additional five minutes, while under observation. The pressure was then rapidly released. There were no visible effects either while the eggs were under pressure or afterwards. Upon subsequent insemination 100 per cent of the eggs yielded good fertilization membranes.

Discussion

The fact that cortical reaction and membrane elevation took place without oxygen in response to certain chemical and physical agents accords well with expectation. For in the experiments of E. B. Harvey (1930) membrane elevation took place in response to the action of sperm in the presence of only minute traces of oxygen, so long as there was sufficient oxygen for the sperm to swim. Indeed Harvey concludes that "If oxygen is necessary for membrane formation, it is an almost infinitesimal amount." Since it is now known that oxygen is not necessary for the egg cortex to respond, doubtless the only factor which prevents its response to sperm in the absence of oxygen is the immotility of the sperm. Additional evidence in this direction is supplied by Loeb's (1915*b*) experiments in which sperm immobilized by treatment with NaCN were incapable of fertilizing the eggs of the sea urchin *Strongylocentrotus purpuratus;* this effect was reversed when the sperm had recovered its motility.

It has been shown in our experiments that suitable chemical treatment under anoxic conditions may lead to cleavage after readmission of air. This eliminates any suggestion that oxygen is necessary for the cortical reaction if cleavage is to take place subsequently. However, the cortical reaction sets in motion processes for which oxygen is necessary. Thus in the absence of oxygen or at very low oxygen tensions no cleavage takes place (Loeb, 1895; E. B. Harvey, 1927; Amberson,

1928). Moreover, in sea urchin eggs the cortical reaction is followed by a striking increase in the rate of oxygen consumption; and this is true regardless of whether membrane elevation is initiated naturally or artificially (see for example Warburg, 1908, 1910; Loeb and Wasteneys, 1911, 1913).

The idea that cleavage furrow formation and amoeboid movement may be different manifestations of the same type of cellular activity has been expressed by Marsland (1936, 1938, 1939) and by Schechtman (1937). It is possible that the amoeboid movement of *Arbacia* eggs in urea solution may perhaps be regarded as a perversion of the activity which would normally be expressed in cleavage. While it seemed at first possible that the oxygen tensions which would just support cleavage might be of the same order of magnitude as those which would support amoeboid movement in *Arbacia* eggs, nevertheless our results show quite clearly that this is not so. Some degree of amoeboid movement occurred even at 0.42 mm. oxygen—a tension far too low for cleavage, although of the same order as that (0.3 mm. oxygen) which will just support some movement of the marine amoeba *Flabellula mira* (Kitching, 1939*b*). It must be remembered, in comparing amoeboid movement with cleavage, that the latter is usually dependent on certain nuclear changes, quite apart from those phenomena of cytoplasmic gelation, such as growth of the asters, which lead to furrow formation.

The influence of high hydrostatic pressure provides an interesting comparison between cleavage of inseminated *Arbacia* eggs (Marsland, 1938), amoeboid movement of *Amoeba dubia* and *Amoeba proteus* (Marsland and Brown, 1936), and amoeboid movement of unfertilized *Arbacia* eggs in urea solution (as recorded in this paper). The effective range of pressure is approximately the same in each case. At 340 atmospheres the ends of the pseudopods of amoebae become spherical, and the organisms finally (at 400 atmospheres) round up entirely; at such pressures inseminated *Arbacia* eggs do not cleave; and amoeboid *Arbacia* eggs stop moving and tend to round up. Amoebae stop moving at 250 atmospheres; unfertilized *Arbacia* eggs in urea solution do not lose their spherical form at 230 atmospheres; and cleavage of inseminated eggs is delayed at pressures below 333 atmospheres. The solation of the cortical gel, which is believed to account for the inhibition of cleavage of inseminated eggs and for stoppage of movement of amoebae under the influence of high hydrostatic pressures, no doubt also accounts for stoppage of amoeboid movement of unfertilized *Arbacia* eggs in urea solution.

It seems rather remarkable that pressures as high even as 680 atmospheres have no apparent harmful effects, at least as regards the cortical

response, on the unfertilized eggs of *Arbacia punctulata,* when applied for short periods (5–10 minutes) of time.

Summary

1. A special technique has been developed for transfer of sea urchin eggs (or other suitable objects) from one solution to another under anoxic conditions while under microscopical observation. The transfer is done with a fine glass loop in an anoxic micromanipulation chamber.

2. Unfertilized eggs of *Arbacia punctulata* were subjected in an atmosphere of pure hydrogen (and water vapor) to treatment with certain chemical and physical agents which in air are known to produce a cortical response, followed usually by membrane elevation.

3. A typical cortical response, usually followed by membrane elevation, was obtained in complete absence of oxygen by treatment with ¼ per cent saponin in sea water, isotonic urea solution, or isotonic sucrose solution, or by micro-puncture. The time relations were the same without oxygen as with it.

4. Transfer of unfertilized *Arbacia* eggs in urea solution, followed by several washes of sea water, all under anoxic conditions, led to cleavage after readmission of air. Thus the treatment which initiates cleavage does not require oxygen.

5. The amoeboid movement of unfertilized *Arbacia* eggs in isotonic urea solution was stopped reversibly in absence of oxygen. Cleavage also is known to require oxygen; but it was found that amoeboid movement took place at an oxygen tension well below that required for cleavage.

6. Unfertilized *Arbacia* eggs in urea solution immediately stopped all movement when the hydrostatic pressure was raised to 340 atmospheres. If the pressure was raised to 230 atmospheres or more before the eggs had begun to lose their spherical shape, no movement occurred. This inhibition of movement was reversed when the pressure was released.

7. Unfertilized eggs which had been compressed to 680 atmospheres for several minutes showed no ill effects, and when inseminated afterwards at atmospheric pressure gave good fertilization membranes.

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